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Lack of B Cell Precursors in Marrow Transplant Recipients With Chronic Graft-Versus-Host Disease

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B cell reconstitution after bone marrow transplantation is slow in patients with chronic graft-vs.-host disease (cGVHD). Could this be secondary to decreased production of B cells in the bone marrow? We determined the relative amount of B cell precursors in the marrow of 26 patients at approximately 1 year after marrow transplant (10 patients with and 16 patients without clinical cGVHD) and 8 normal adult controls. In the controls (median), 3.1% of all marrow mononuclear cells were B cell precursors. The patients without cGVHD tended to have higher than normal percents of B cell precursors (median 6.5%; the difference from normal adults was not significant). In contrast, the patients with cGVHD had barely detectable B cell precursors (median 0.2%; the difference from normal adults was significant, $P = 0.004$). Therefore, delayed reconstitution of B cells in patients with cGVHD appears to be due at least in part to decreased B cell production by the marrow.

Key words: bone marrow transplantation, chronic graft-vs.-host disease, B-lymphocytes

INTRODUCTION

Humoral immunity of marrow transplant recipients is deficient for 2 years or more following the transplant, particularly in patients with chronic graft-vs.-host disease (cGVHD) [1–5]. The pattern of quantitative B cell development after uncomplicated marrow grafting resembles the pattern of quantitative B cell development in early life: during the fetal/neonatal/infant periods, blood B cell counts rise from zero to values exceeding those of normal adults [6–9]. Similarly, during the first 1–2 years after transplant without cGVHD, blood B cell counts usually rise from barely detectable to values exceeding those of normal adults. However, in transplant recipients with cGVHD, the quantitative reconstitution is slower; some patients with cGVHD have lower blood B cell counts than seen in normal adults even at 2 years after transplant [10,11].

In mammals, naive B cells are produced exclusively in bone marrow [12,13]. Among human marrow B-lineage cells, defined by the expression of CD19 and/or CD20 [14], those expressing CD34 and/or CD10 appear to constitute the proliferative pool of B cell precursors [15–17].

We tried to answer the following question: Is the quantitative deficiency of B cells in patients with cGVHD due to decreased production of naive B cells, in which case the amount of $CD34^+$ and/or $CD10^+$ B cell precursors should be low, or due to increased destruction of B cells, in which case the amount of $CD34^+$ and/or $CD10^+$ B cell precursors might be high? $CD34^+$ and/or $CD10^+$ B cell precursors in the marrow of patients with cGVHD were barely detectable. Therefore, insufficient production of naive B cells contributes to or accounts for the slow tempo of B cell reconstitution in patients with cGVHD.

MATERIALS AND METHODS

Patients and Donors

Twenty-six patients were studied at approximately 1 year after marrow grafting. Most of the patients' clinical data are presented in Table I. Recipients of allogeneic grafts were typically conditioned with cyclophosphamide (120 mg/kg) plus either fractionated total body irradiation (12 Gy) or busulfan (16 mg/kg) [18,19]. Three autologous transplant recipients were conditioned in a manner similar to that of the allogeneic transplant recipients and three

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TABLE I. Patient Characteristics*

Patient group	1 year after auto-transplant	1 year after allo-transplant, no cGVHD	1 year after allo-transplant, with cGVHD
Number of patients studied	6	10	10
Median postransplant day of B cell/B cell precursor assessment	354	363	370
[25th–75th percentile]	[323–361]	[349–374]	[356–395]
(range)	(266–500)	(248–404)	(336–532)
Number of patients with pre-transplant diagnoses other than hematologic malignancies	1 (17%) (breast cancer)	1 (10%) (aplastic anemia)	1 (10%) (aplastic anemia)
Donor-host histocompatibility[a] (number of patients) [%]	Auto (6) [100%]	Id sib (6) [60%], MUD (3) [30%], MMD (1) [10%]	Id sib (6) [60%], MUD (2) [20%], MMD (2) [20%]
Number of patients with clinical cGVHD at any time prior to B cell/B cell precursor assessment[b]	0 (0%)	4 (40%)	10 (100%)
Number of patients with active clinical cGVHD at the time of B cell/B cell precursor assessment	0 (0%)	0 (0%)	10 (100%)
Number of patients treated with immune suppressants at the time of B cell/B cell precursor assessment[c]	0 (0%)	4 (40%)	6 (60%)
Number of patients treated with IVIG at the time of B cell/B cell precursor assessment	0 (0%)	2 (20%)	3 (30%)

*Intravenous immunoglobulin (usually 500 mg/kg monthly).

[a]Auto = autologous, Id sib = HLA-A,B&DR-identical sibling, MUD = HLA-A,B&DR-matched unrelated donor, MMD = mismatched donor in at least one HLA-A,B or DR antigen.

[b]Includes both patients with active cGVHD as well as those with extinct cGVHD (those who no longer had cGVHD at the time of B cell/B cell precursor assessment).

[c]Usually prednisone (0.5–1.0 mg/kg every other day) plus cyclosporine (approximately 6 mg/kg every other day per os) [23]. The reasons for giving immunosuppressive drugs to the four of the ten allograft recipients without active cGVHD at 1 year after transplant were: treatment of past cGVHD that was no longer active (2 patients), prophylaxis against cGVHD in 1 patient who had been deemed to be at high risk for developing cGVHD, and treatment of idiopathic lymphocytic pneumonitis in 1 patient. The reasons for not giving immunosuppressive drugs to the four of the ten patients with active cGVHD at 1 year after transplant were: prior to the annual exam only limited cGVHD was diagnosed (3 patients), and 1 patient with extensive cGVHD refused immunosuppressive drugs.

autotransplant recipients were conditioned with BCNU (600 mg/m^2), etoposide (2.4 g/m^2), and cyclophosphamide (1.8 g/m^2) [20]. Bone marrow of two autograft recipients had been purged with either 4-hydroxyperoxycyclophosphamide or anti-B cell antibodies plus complement; all remaining patients received unpurged marrow. Allograft recipients typically received GVHD prophylaxis with methotrexate (days 1, 3, 6, and 11) and cyclosphorine (daily during the first 6 months after BMT) [21]. Acute GVHD was usually treated with high-dose corticosteroids [22]. All but one patient studied were in remission at the time of B cell assessment. The one exception was a patient with acute myelogenous leukemia allotransplanted in complete remission who at the time of B cell evaluation had an isolated chloroma in the proximal radius; pelvic bone marrow and peripheral leukocyte count and differential were normal.

Peripheral blood (n = 22) and marrow (n = 8) from adult volunteers (ages 20–50) were studied to provide normal control data.

Specimen Procurement and Fractionation

All the procedures were approved by the FHCRC Institutional Review Board and written consent was always obtained.

Bone marrow aspirate was obtained from a posterior iliac crest; to minimize dilution with blood, only a small amount of marrow (~1 ml) was aspirated. Blood was taken by venipuncture or from a central venous catheter.

Mononuclear cells (MNC) were separated by density-gradient centrifugation using Ficoll-Hypaque (1,077 kg/m^3).

Flow Cytometry

Blood B cells were enumerated as previously described [11]. Briefly, the percent of MNC expressing CD19 and/

or CD20 was determined by flow cytometry. Absolute number of B cells per unit blood volume (Abs_B) was determined as

$$Abs_B = (Abs_{Ly} + Abs_{Mo}) \times \%B \text{ cells of all MNC} / 100,$$

where Abs_{Ly} = absolute lymphocyte count, and Abs_{Mo} = absolute monocyte count.

For the determination of B cell precursors, antibodies linked to 3 different fluorochromes (fluorescein isothiocyanate [FITC], phycoerythrin [PE], and peridinin chlorophyll protein reagent [PerCP]) were utilized concurrently. The fluorochrome-conjugated antibodies were purchased from Becton-Dickinson, San Jose, CA, except for CD3-PE and CD16-PE which were purchased from AMAC/Immunotech, Marseille, France. Bone marrow MNC were simultaneously stained with CD34-FITC, CD10-FITC, CD3-PE, CD13-PE, CD14-PE, CD16-PE, CD33-PE, CD19-PerCP, and CD20-PerCP. Contaminating erythrocytes were lyzed with ammonium chloride. Samples were not fixed; they were kept in the dark at 4°C before flow cytometry which was performed within 4 hr after staining. A FACScan flow cytometer (Becton-Dickinson) was used for data acquisition. All cells within a forward × side scatter gate including those with lymphocyte, blast, and monocyte scatter profiles were analyzed (Fig. 1, upper left dotplot, region R1). B-lineage cells were quantitated using a fluorescence-2 (PE) × fluorescence 3 (PerCP) plot (Fig. 1, middle left dotplot, region R2); they were defined as ($CD19^+$ or $CD20^+$) and ($CD3^-$ or $CD13^-$ or $CD14^-$ or $CD16^-$ or $CD33^-$) MNC. To properly draw the region border around the B-lineage cells, a control fluorescence-2 × fluorescence-3 plot was examined, i.e., a plot derived from the same marrow cells but stained with any FITC-labeled antibody (usually CD34-FITC), CD3-PE, CD13-PE, CD14-PE, CD16-PE, CD33-PE, and isotypic control-PerCP (Fig. 1, upper right panel). For the quantitation of B cell precursors, a fluorescence-1 (FITC) histogram was created of only B-lineage cells (Fig. 1, middle right panel). The percent of the $CD10^+$ and/or $CD34^+$ B cell precursors was calculated with regard to the isotypic control (i.e., same marrow cells but stained with isotypic control-FITC, CD3-PE, CD13-PE, CD14-PE, CD16-PE, CD33-PE, CD19-PerCP, and CD20-PerCP).

No attempt was made to quantitate subpopulations of the $CD10^+$ and/or $CD34^+$ B cell precursors (e.g., $CD34^+CD10^-$, $CD34^+CD10^+$ or $CD34^-CD10^+$ B cell precursors).

Determination of Marrow Cellularity and Quantitation of Marrow MNC

Bone marrow cellularity was estimated from standard decalcified biopsy sections or aspirated particle sections stained with hematoxylin and eosin. Cellularity was expressed as percent expected normal cellularity. For example, 50% fat:50% hematopoietic cells was expressed as 100% cellularity, and 75% fat:25% hematopoietic cells was expressed as 50% cellularity. Percent MNC of all nucleated cells was determined from standard May-Grunwald/Giemsa-Romanowski-stained smears of marrow aspirates. The following cells were counted as MNC: proerythroblasts, basophilic, polychromatophilic and eosinophilic normoblasts, lymphocytes, lymphoblasts, myeloblasts, and promyelocytes. (These cells fall within the MNC gate on forward × side scatter plots [24].)

Statistics

Medians and percentiles rather than means and standard deviations were used since the data did not show normal distribution. Non-parametric Mann-Whitney U test (2-tailed) was used for comparisons of the quantity of B cells or B cell precursors between patient/normal groups.

RESULTS

Circulating B Cells Are Low in Patients With cGVHD

As shown in Figure 2, at approximately 1 year after transplant, autograft recipients had high B cell counts (median 382 × 10^6/l, range 130–1,128), allograft recipients without clinical cGVHD tended to have near-normal B cell counts (median 155 × 10^6/l, range 3–990), and allograft recipients with clinical cGVHD had low B cell counts (median 99 × 10^6/l, range 8–315). Normal adult median was 178 × 10^6/l (range 90–475). The difference between the autograft recipients and the normals was significant ($P < 0.001$). The difference between the allograft recipients with cGVHD and the normals was also significant ($P < 0.001$). The difference between the allograft recipients with cGVHD and the allograft recipients without cGVHD was not significant ($P = 0.20$), probably due to small sample size and high variability among the allograft recipients without cGVHD.

Marrow B Cell Precursors Are Low in Patients With cGVHD

As shown in Figure 3, $CD34^+$ and/or $CD10^+$ B cell precursors constituted (median) 3.1% of marrow MNC in normal adults (range 1.0–26.2), 7.6% of marrow MNC in autograft recipients (range 0.5–17.8), and 5.8% of marrow MNC in allograft recipients without cGVHD (range 0.1–31.2). The differences between autotransplant recipients and normal adults and between allotransplant recipients without cGVHD and normal adults were insignificant ($P = 0.29$ and $P = 0.25$, respectively). In contrast to the autograft recipients and allograft recipients without cGVHD, $CD34^+$ and/or $CD10^+$ B cell precursors were extremely sparse in patients with cGVHD, accounting for

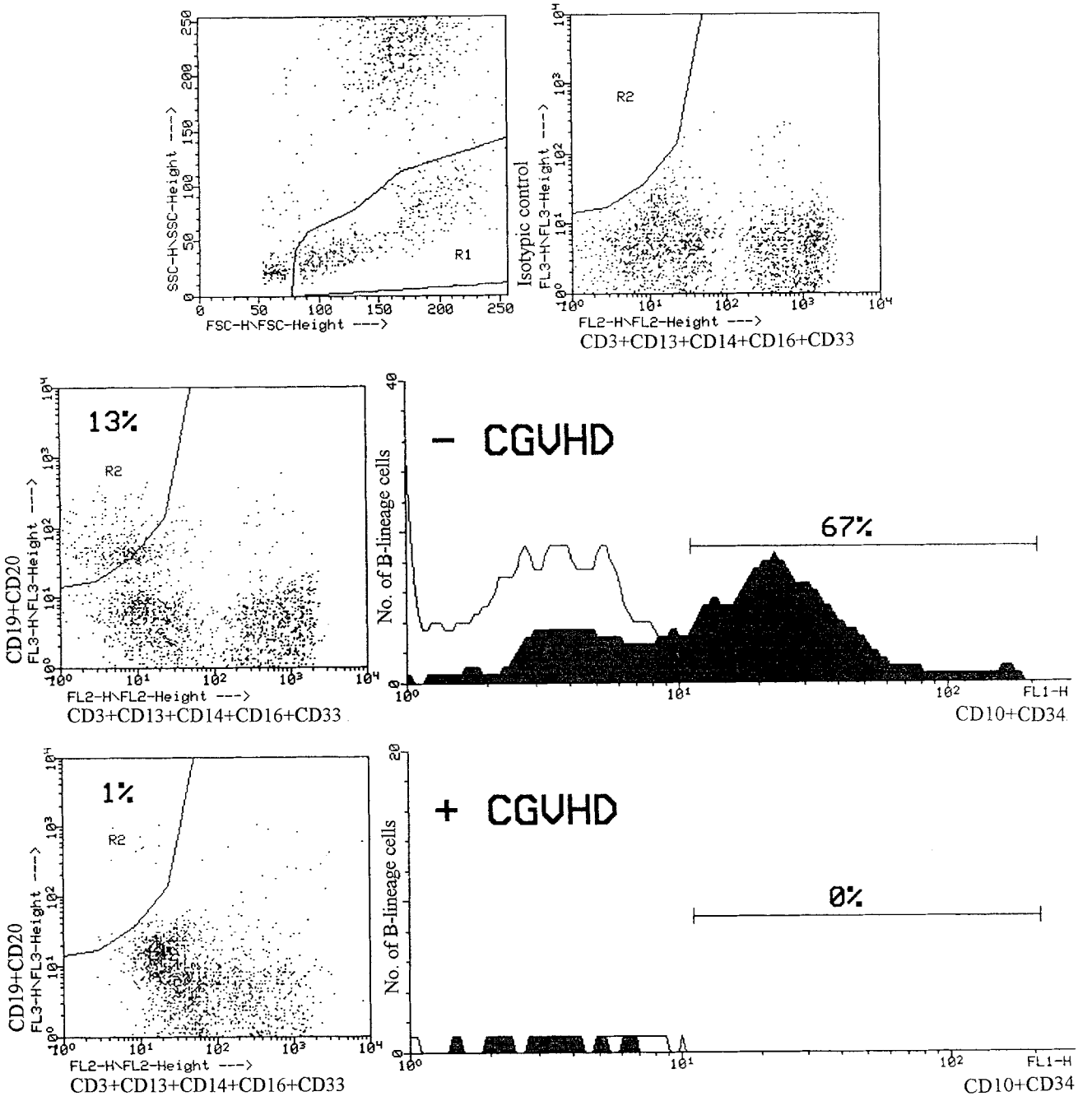

Fig. 1. Abundant CD34⁺ and/or CD10⁺ B cell precursors were detected in the marrow of the allo-BMT recipient without clinical chronic GVHD ("-CGVHD") whereas no B cell precursors were detected in the marrow of the allo-BMT recipient with clinical chronic GVHD ("+CGVHD"). None of these two patients were treated with immunosuppressive drugs for at least 8 weeks prior to the B cell assessment. Method: After cell staining and data acquisition on FACScan as described in Materials and Methods, all mononuclear cells were gated using a forward scatter (FSC) vs. side scatter (SSC) plot (upper left dotplot, region R1). Then, B-lineage cells were gated and enumerated using a fluorescence-2 (PE) vs. fluorescence-3 (PerCP) plot (middle left and lower left dotplots, region R2) with regard to a control fluorescence-2 vs. fluorescence-3 plot (upper right dotplot). Then, a fluorescence-1 (FITC) histogram was created of only B-lineage cells (middle right and lower right panels) and the percentage of the CD10⁺ and/or CD34⁺ cell precursors (of all B-lineage cells) was calculated with regard to the isotypic control (the line with no area-under-the-curve shading). Example of calculation: In the allo-BMT recipient without chronic GVHD (middle dotplot and histogram), 13% of all marrow mononuclear cells were B-lineage cells and 67% of the B-lineage cells were B cell precursors. Therefore, 13 × 67/100 = 8.7% of all marrow mononuclear cells were B cell precursors.

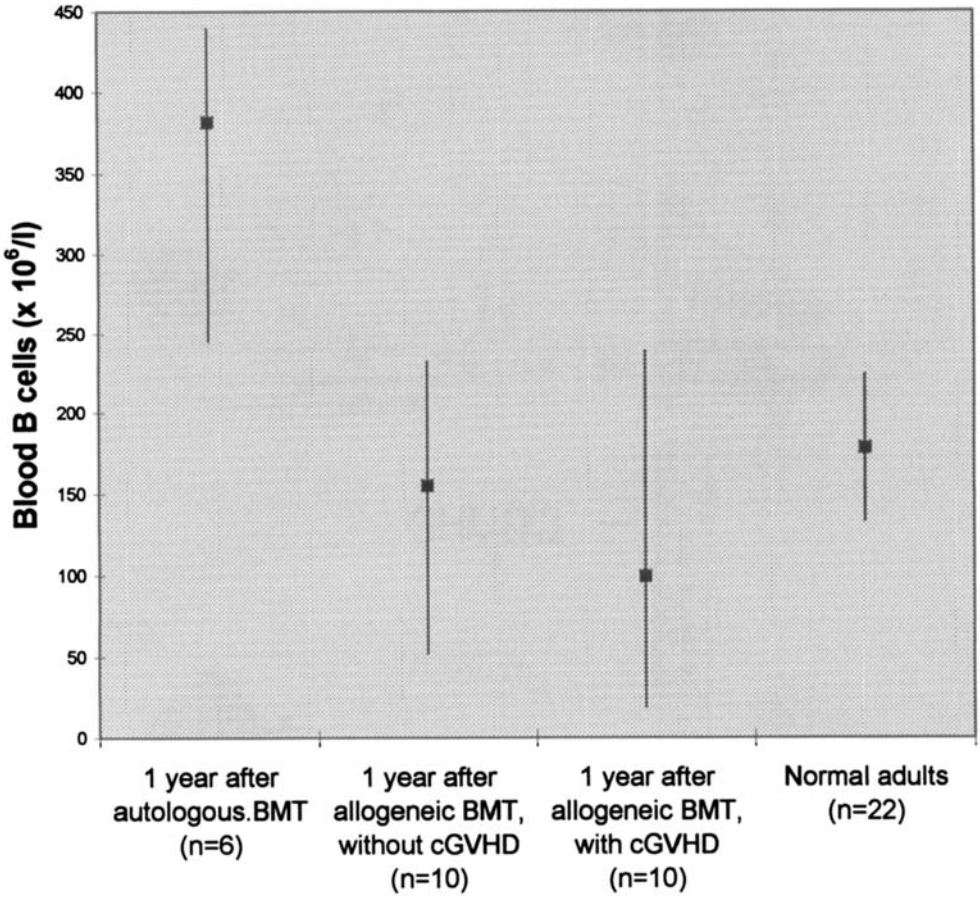

Fig. 2. Blood B cell counts in patients at 1 year after bone marrow transplant as well as in normal adults. Data are displayed as medians (squares) and 25–75th percentiles (vertical lines).

only (median) 0.2% of marrow MNC (range 0–37.4). The difference between normal adults and patients with cGVHD was significant ($P = 0.004$). The difference between the allograft recipients with cGVHD and the allograft recipients without cGVHD was nearly significant ($P = 0.07$).

Next we wanted to rule out the possibility that the cGVHD patients had a normal absolute number of marrow $CD34^+$ and/or $CD10^+$ B cell precursors despite the low percentage of marrow $CD34^+$ and/or $CD10^+$ B cell precursors. This could be true if either the marrow cellularity or the percent of MNC (of all marrow nucleated cells) was increased in the cGVHD patients. The median marrow cellularities in normal adults, allograft recipients with cGVHD, allograft recipients without cGVHD, and autograft recipients were similar (95, 100, 100, and 100%, respectively); there was no significant difference between any two of these four groups. The median percents of MNC (of all marrow nucleated cells) in allograft recipients with cGVHD, allograft recipients without cGVHD, and autograft recipients were also similar (53, 42, and 58%, respectively); there was no significant difference between any two of these three groups; the median percent of mononuclear cells in the adult normals was not determined. Therefore, the absolute marrow B cell precursor count, which cannot be directly assessed, was very low in the patients with cGVHD.

DISCUSSION

In the absence of cGVHD, the tempo of quantitative B cell reconstitution after marrow grafting is approximately the same as in early life, leading to an overshoot of blood B cell counts above normal adult range by 1–2 years posttransplant [7–11]. The data presented here confirm previous observations [10,11] that in the presence of cGVHD, quantitative B cell reconstitution tends to be slower. Autograft recipients, who should have had no cGVHD by definition, had higher than normal adult B cell counts, whereas allograft recipients with clinical cGVHD still had lower than normal adult B cell counts at approximately 1 year after transplant. Allograft recipients without clinical cGVHD tended to have B cell counts intermediate between those of the autograft recipients and those of

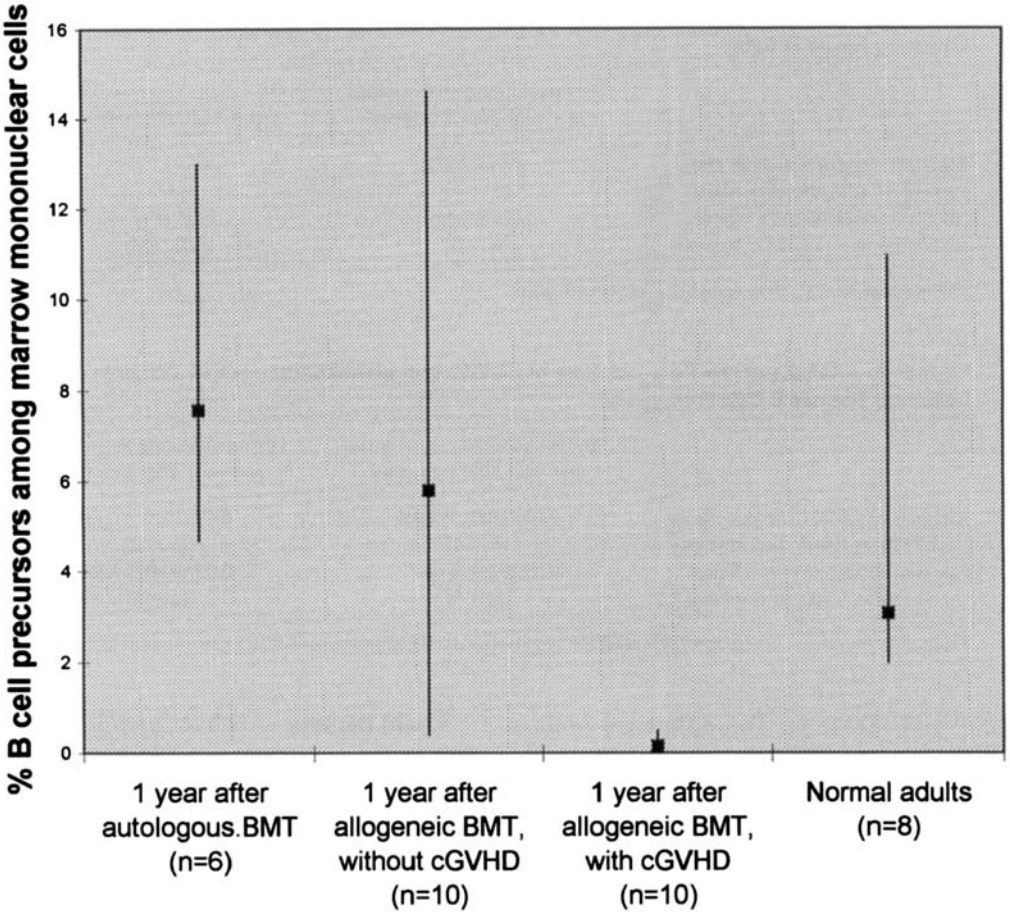

Fig. 3. Relative amount of CD34$^+$ and/or CD10$^+$ B cell precursors in the bone marrow of patients at 1 year after bone marrow transplant as well as in normal adults. Data are displayed as medians (squares) and 25–75th percentiles (vertical lines).

the allograft recipients with clinical cGVHD, perhaps reflecting the possibility of subclinical cGVHD.

Previous studies on marrow B-lymphopoiesis after marrow grafting showed that compared to normal adults, B cell precursors are abundant at around 1 year after transplant [25–28]. The vast majority of patients in these studies were those without cGVHD. Patients without cGVHD had abundant B cell precursors also in this study. This is reminiscent of normal B cell development in early life: marrow B cell precursors are abundant in infancy [9], presumably in order to provide the developing immune system with a wide spectrum of naive B cells.

To our knowledge, this is the first study of marrow B cell precursors in patients with cGVHD. In contrast to patients without cGVHD, CD34$^+$, and/or CD10$^+$ B cell precursors were sparse in patients with cGVHD. Because these cells constitute the proliferative pool of marrow B-lineage cells [15–17], we interpret the relative lack of these cells as insufficient production of naive B cells in patients with cGVHD. This probably accounts for the observed delay in the reconstitution of blood B cell counts. Nevertheless, possible contribution of decreased proliferation of B cells in lymph nodes, spleen, or mucosa-associate lymphoid tissue or increased destruction of B cells cannot be excluded. However, these mechanisms are unlikely based on the following: Despite that our patients with cGVHD had extremely low amounts of B cell precursors in the marrow (Fig. 3), their blood B cell counts were lower than normal but not extremely low (Fig. 2). If the extremely few B cells generated in the marrow did not proliferate in the peripheral lymphoid organs or were being destroyed at a high rate, the blood B cell counts should be extremely low.

Could our results be influenced by the wide range of days after transplant when patients' B cells/B cell precursors were studied? Assuming blood B cell counts keep increasing till at least 18 months after grafting [10,11], our conclusions could be wrong if the allograft recipients with cGVHD were evaluated at an earlier time-point after grafting than the allograft recipients without cGVHD. The opposite was true: allograft recipients with cGVHD were evaluated slightly later than allograft recipi-

TABLE II. Analysis of the Potential Role of cGVHD and Immunosuppressive Drugs in Lowering Blood B Cells*

	On immunosuppressive drugs at 1 year after transplant	Off immunosuppressive drugs at 1 year after transplant
Allograft recipients without clinical cGVHD at 1 year after transplant	48 (3–147) [n = 4]	305 (155–390) [n = 6]
Allograft recipients with clinical cGVHD at 1 year after transplant	47 (8–315) [n = 6]	205 (8–285) [n = 4]

*Values represent median (range) blood B cell count.

TABLE III. Analysis of the Potential Role of cGVHD and Immunosuppressive Drugs in Lowering Marrow B Cell Precursors*

	On immunosuppressive drugs at 1 year after transplant	Off immunosuppressive drugs at 1 year after transplant
Allograft recipients without clinical cGVHD at 1 year after transplant	2.95 (0.00–14.60)* [n = 4]	6.47 (0.40–31.15) [n = 6]
Allograft recipients with clinical cGVHD at 1 year after transplant	0.08 (0.00–4.60) [n = 6]	0.08 (0.00–37.35) [n = 4]

*Values represent median (range) percent $CD34^+$ and/or $CD10^+$ B cell precursors of all marrow mononuclear cells.

ents without cGVHD (Table I). The difference in days posttransplant was statistically insignificant ($P > 0.20$). Therefore, the difference in the timing of the "one-year-posttransplant" evaluation should not account for the trend towards low amounts of circulating B cells and marrow B cell precursors in patients with cGVHD.

Since patients with cGVHD are usually treated with immunosuppressive drugs, it has been difficult to discern whether the relative lack of B cells is due to cGVHD itself or due to the immune suppressants. In an attempt to address this problem, we split our allotransplant recipients into those on vs. off immune suppressants at the time of B cell/B cell precursor evaluation (Tables II and III; for the list of reasons for giving immune suppressants to some patients without cGVHD and not giving immune suppressants to some patients with cGVHD, see Table I, footnote[c]). The data from Tables II and III suggest that both immune suppressants and cGVHD may play a role. However, the lower B cell/B cell precursor amounts in patients on than in patients off immune suppressants does not necessarily mean that immune suppressants delay B cell reconstitution. Treatment with immune suppressants may be just a marker of patients with more severe cGVHD. Only one of our patients with extensive cGVHD (the one presented in Fig. 1, bottom) was not treated with immune suppressants due to refusal. In contrast to allograft recipients without cGVHD and off immune suppressants, this patient had barely detectable blood B cells (8×10^6/l) and undectable marrow B cell precursors. In summary, we could not solve whether cGVHD itself, immune suppressants, or both contributed to the quantitative deficiency of B cells/B cell precursors; however, a role of cGVHD itself appeared likely.

Could the lack of $CD34^+$ and/or $CD10^+$ B cell precursors in patients with cGVHD be caused by an immune attack against marrow stromal cells supporting the proliferation and differentiation of B cell precursors or against the B cell precursors? The former case is conceivable since posttransplant stromal cells are of host origin whereas posttransplant lymphocytes in T-replete graft recipients are usually of donor origin [29–32]. Graft-vs.-stroma reaction has been well documented in transplant recipients with prolonged or relapsing cytopenias and acute GVHD [33,34]. Graft-vs.-stroma reaction also appears to exist in at least some cytopenic patients with chronic GVHD [33,35]. Autoimmune attack of donor-derived lymphocytes against donor-derived B cell precursors is also conceivable. Several cases of donor-derived lymphocytes attacking donor-derived red blood cells or platelets have been reported (reviewed in [36]). However, it is not known whether the data on erythroid precursos, myeloid precursors, red blood cells, and platelets can be extrapolated on B cell precursors.

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